VIER

Sensors and Actuators B 38–39 (1997) 38–41

SENSORS AND ACTUATORS B CHEMICAL

Bioengineering of silicon nitride

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ve functionalization of silicon nitride with biomolecules by light-dependent processes has been investigated. Aryldiazirin-based obilization procedures are used to achieve covalent biomolecule binding. Experimentally facile processes applied include the steps: (i) adsorptive coating of the surface with photolabel-bearing reagents or photolabel-functionalized biomolecules; (ii) of the coated surface to activating light (350 nm); and (iii) removal of excess reagent or functionalized biomolecule. The extent of ent binding to silicon nitride depends on the time of light exposure as well as on the amount of photoreagent applied to the surface. in is immobilized by photolinker polymer-mediated procedures, and antibody-derived F(ab′) fragments are covalently immobilized nitride (45–50 fmol mm^{-2}) with a low-molecular-weight crosslinker. Biomolecule binding is monitored by fluorescein-labelled ding and by tracing radiolabelled proteins, respectively. Photoimmobilized streptavidin retains ligand binding activity, and immu- remain biologically active. Mask-assisted photopatterning on silicon nitride is achieved and patterned structures are resolved by ce microscopic imaging of photobonded diazirin-derivatized bovine serum albumin.

Photoimmobilization; Aryldiazirin; Silicon nitride; Bioengineering; Oriented immobilization

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t years, considerable attention has been focused on c materials which combine the properties and spec- iomolecules with the intrinsic physical and chem- cteristics of materials. For the design of biosensors, le, emphasis has been placed on the bioengineering ls, which are attractive due to their signal-trans- perties (e.g., electrical conductivity, optical prop- ustic wave transmission) and, in addition, permit nmobilization of sensing molecules at the material ilicon nitride is known as a hard material with l wear resistance and high temperature stability se of its excellent properties as an etch mask (pho- hy) and as a diffusion barrier, silicon nitride is used in the micromachining of three-dimensional in silicon [2]. Many applications in microelec- lize silicon nitride as an electrically insulating 3]. Optical waveguidance (refractive index ualifies silicon nitride as an efficient signal-trans- terial for optical sensors [4]. Moreover, silicon mic offers the attractive features of biocompati- an elastic modulus similar to that of bone renders the material amenable for dental repair and osseous reconstruction [5]. This study describes a facile method for covalent immobilization of biomolecules on silicon nitride.

2. Materials and methods

Silicon wafers (orientation [100], p-type) or glass surfaces having thermal nitride layers were diced (8 mm × 8 mm, 5 mm × 5 mm). ImmunoPure anti mouse IgG $F(ab')_2$ and ImmunoPure mouse IgG were purchased from Pierce; $F(ab')_2$ fragments of anti-PSA antibody (a monoclonal mouse antibody, elicited against the prostate-specific antigen) were obtained from Hoffman-La Roche, Basel, Switzerland; BSA (bovine serum albumin) was from Sigma. The photolinker polymer T-BSA (bovine serum albumin derivatized with 3-(trifluoromethyl)-3-(*m*-isothiocyanophenyl) diazirin was prepared according to published procedures [6]. *N*-[*m*-(3-(trifluoromethyl) diazirin-3-yl]-phenyl)-4-maleimido butyramide (MAD) was synthesized according to Collioud et al. [7]. Radiolabelled formaldehyde ($[^{14}C]$-HCOH, 53 mCi $mmol^{-1}$) was from New England Nuclear and PD 10 (Sephadex G-25M) columns were pur-

For patterning purposes a nickel photomask with 20 μm slits separated by 180 μm spacings was purchased from Towne Laboratories Inc., Somerville, NJ. Surface imaging was carried out on a standard scanning force microscope, equipped with a home-built liquid cell (Universal, Park Scientific Instruments, Sunnyvale, CA). Surface fluorescence was detected with the Axiovert 35 microscope (Zeiss, Germany).

2.1. Pre-treatment of diced silicon nitride

Silicon nitride coated chips were rinsed with (and sonicated in) acetone and isopropanol, and dried under vacuum.

2.2. Preparation of [^{14}C]-radiolabelled mouse IgG and anti-PSA F(ab')$_2$ fragments

Mouse IgG (1 mg in 500 μl 100 mM sodium phosphate buffer, pH 6.8) was transferred to 0.1 M 4-(2-hydroxyethyl)-piperazine-1-ethane-sulfonic acid (HEPES) buffer pH 7.5 by chromatography on PD 10. Then 29.7 μl (1 μmol, 53 μCi) [^{14}C]-formaldehyde and 65 μmol sodium cyanoborohydride were added to the collected protein (890 μg in 1.5 ml HEPES buffer) and the reaction mixture was stirred for 4 h at ambient temperature in the dark. [^{14}C]-methylated mouse IgG was separated from the reaction mixture by chromatography on PD 10 in 0.1 M sodium phosphate buffer, pH 6.8. Specific radioactivity was determined by scintillation counting and protein concentration was determined by measuring the 280 nm absorption ($A_{280}^{1\%} = 14.0$). Radiolabelling of anti-PSA F(ab')$_2$ fragments was carried out analogously, and modified antibodies were stored at −20°C in 0.1 M sodium phosphate buffer, pH 6.8.

2.3. T-BSA-mediated photoimmobilization of streptavidin and binding of fluorescein-labelled biotin on silicon nitride

Streptavidin (0.25 μg) and 1.0 μg of T-BSA in 25 μl PBS buffer (phosphate-buffered saline consisting of 150 mM NaCl, 5 mM sodium phosphate buffer, pH 7.4) were applied to each chip. After drying under vacuum at 37°C, chips were irradiated for 20 min with the Stratalinker light source. Surfaces were then washed with PBS containing 0.02% Tween 20 (3×0.5 ml/chip), bidistilled water (3×0.5 ml/chip). Identically treated but not irradiated samples served as controls. Fluorescein-labelled biotin (25 μl in PBS buffer, 1:30 diluted 1 mg ml^{-1} stock solution) was added and chips were incubated overnight at room temperature. Following washing with PBS buffer (5×1 ml), chips were inspected by fluorescence microscopy.

2.4. Preparation of anti-mouse IgG F(ab') fragments and [^{14}C]-radiolabelled F(ab') fragment of anti-PSA antibodies

The F(ab')$_2$ fragment of anti-mouse IgG (100 μg in 500 μl of 100 mM sodium phosphate buffer, pH 6.8) was treated with 1 mM dithiothreitol for 1 h at 37°C. F(ab') fragments were separated from the reaction mixture by chromatography on a PD10 column in 0.1 M sodium acetate, 0.5 M sodium chloride, 1 mM EDTA, pH 5.0. The protein concentration was determined by measuring absorption at 280 nm ($A_{280}^{1\%} = 14$). [^{14}C]-radiolabelled F(ab') fragments of anti-PSA antibody were prepared analogously.

2.5. Photochemical immobilization of N-[m-(3-(trifluoromethyl) diazirin-3-yl]-phenyl)-4-maleimidobutyramide (MAD) and oriented binding of F(ab') fragments

Crosslinker-concentration-dependent binding of F(ab') fragments to silicon nitride was investigated by applying various amounts of MAD. Diced chip surfaces were coated with 2 to 125 nmol MAD and irradiated for 20 min as described above. Binding of [^{14}C]-radiolabelled F(ab') fragment was monitored with freshly prepared F(ab') fragments derived from monoclonal mouse anti-PSA antibodies. Retained radioactivity was quantified by scintillation counting.

2.6. Immunoactivity of oriented immobilized anti-mouse IgG F(ab') fragments

The immunoactivity of oriented immobilized goat anti-mouse IgG F(ab') fragments was analysed by immunocomplexation with [^{14}C]-radiolabelled mouse IgG. F(ab')-fragment-modified chips were saturated with 1% BSA in PBS buffer for 1 h at 37°C. After washing three times with bidistilled water, 2.0 μg of [^{14}C]-radiolabelled mouse IgG in 45 μl 0.1 M sodium phosphate buffer, pH 6.8 was applied per chip, and the samples were incubated for 30 min at 37°C. Identically treated MAD-derivatized surfaces were prepared as control samples. In these samples the incubation with F(ab') fragments was not carried out (−F(ab')). A further set of control samples included surface coating with MAD (but no light exposure) and subsequent addition of F(ab'). Modified chips were washed with water three times and retained radioactivity was quantified by scintillation counting.

3. Results and discussion

Photolinker polymer (T-BSA)-mediated light-dependent immobilization of streptavidin to silicon nitride surfaces was successful. Detection of fluorescein-labelled biotin on photobonded streptavidin revealed retention of biological activity (Fig. 1, T-BSA, +light). Control samples, not exposed to light, showed minor residual fluorescence (Fig. 1, T-BSA, −light). Covalent, light-dependent and oriented immobilization of F(ab') fragments to silicon nitride using MAD as crosslinking agent is documented in Fig. 2. MAD dissolved in ethanol was spread as a thin film on silicon nitride surfaces. Covalent binding of the crosslinker was effected by light exposure. The availability of reactive maleimides was sub-

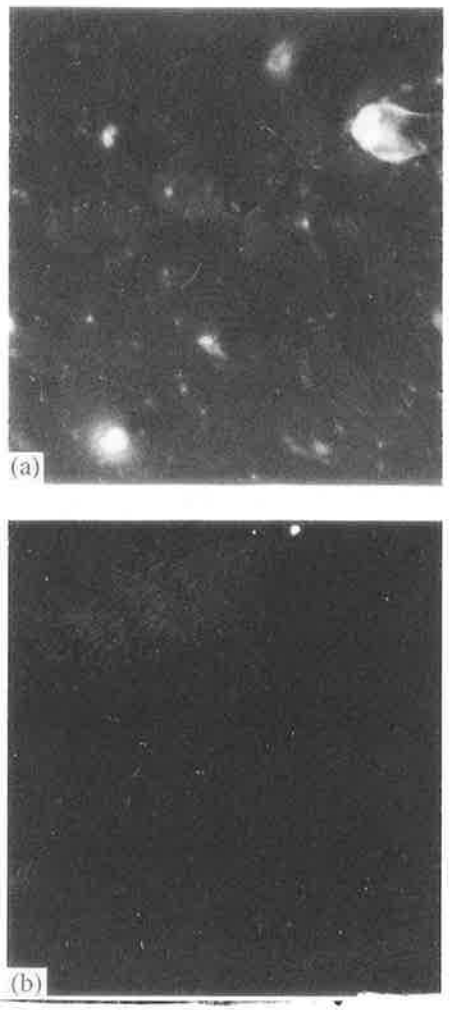

Fig. 1. T-BSA-mediated immobilization of streptavidin to ...nitride and binding of fluorescein-labelled biotin. Streptavidin (0.25 μg) and T-BSA (1.0 μg) in 25 μl PBS buffer were applied to each chip. (a) Following light activation (T-BSA, +light) and washing as described in Section 2, fluorescein-labelled biotin was added and incubated overnight at room temperature. (b) A control sample was identically treated but not irradiated (T-BSA, −light). Surface fluorescence was detected with an Axiovert 35 microscope.

stantiated by subsequent F(ab′) fragment binding. [^{14}C]-radiolabelled F(ab′) fragments were covalently immobilized on MAD-derivatized surfaces. With low MAD concentrations applied to the surface, the observed binding pattern followed the surface concentration of applied photocrosslinker. Saturation of reagent binding occurred above 400 pmol (applied) MAD/mm^2 (Fig. 2). The surface density of thermochemically immobilized F(ab′) saturated at 48 fmol mm^{-2}, which corresponds approximately to one monolayer of F(ab′) molecules. Results shown in Fig. 3 demonstrate that oriented immobilized F(ab′) fragments retain their biological activity. The immunoreactivity of surface-immobilized anti-mouse F(ab′) fragments was monitored by binding of [^{14}C]-radiolabelled mouse IgG. Radioactivity recovered after immunocomplexation (and exhaustive washing to remove physically adsorbed antigen) indicated immunoreagent binding to oriented immobilized F(ab′) fragments of anti-mouse IgG antibody (control samples: MAD coating,

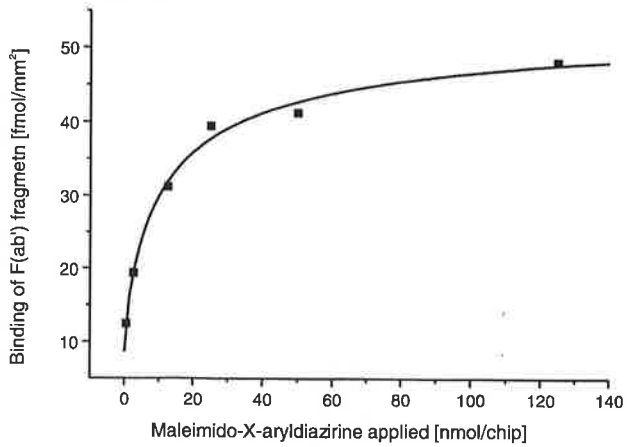

Fig. 2. Crosslinker (MAD)-concentration-dependent immobilization of F(ab′) fragments. Diced silicon nitride surfaces (8 mm×8 mm) were coated with 2–125 nmol MAD and irradiated for 20 min. After several washing steps as described in Section 2, freshly prepared [^{14}C]-radiolabelled F(ab′) fragments of anti-PSA antibody (2.0 μg per chip) were thermochemically coupled to modified chip surfaces. The surface density of F(ab′) fragment saturated at 48 fmol mm^{-2}.

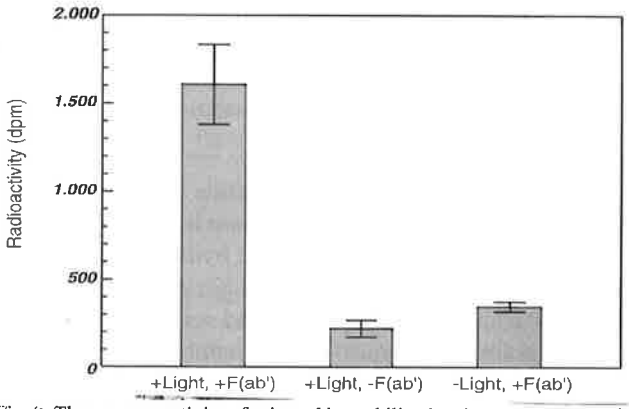

Fig. 3. The immunoactivity of oriented immobilized anti-mouse IgG F(ab′) fragments was analysed by immunocomplexation with [^{14}C]-radiolabelled mouse IgG. 2.0 μg of [^{14}C]-radiolabelled mouse IgG in 45 μl 0.1 M sodium phosphate buffer, pH 6.8 was applied per F(ab′) modified chip (+light, +F(ab′)). Identically treated MAD-derivatized surfaces were prepared as control samples (+light, −F(ab′)). A further set of control samples included surface coating with MAD, but no light exposure, and subsequent addition of F(ab′) (−light, +F(ab′)).

no light exposure, incubation with F(ab′); MAD coating with light exposure, without addition of F(ab′)). This experiment supports the finding that F(ab′) immobilization strictly depends on the availability of MAD on the surface, and indicates immunospecific antigen binding. Light-dependent biomolecule binding to silicon nitride is further demonstrated by mask-assisted photoprinting (Fig. 4). After photoimmobilization of surface-spread T-BSA and extensive washing, the photolinker polymer was detected by atomic force microscopy in those areas that were exposed to light. Smooth surfaces were recorded in the wide 'spacer' areas, indicating the absence of photoimmobilized protein.

One of the major challenges in bioengineering of materials is the necessity for essentially irreversible attachment of the biologically active molecule to material surfaces in a fashion that favours retention of biological activity. Electrochemical

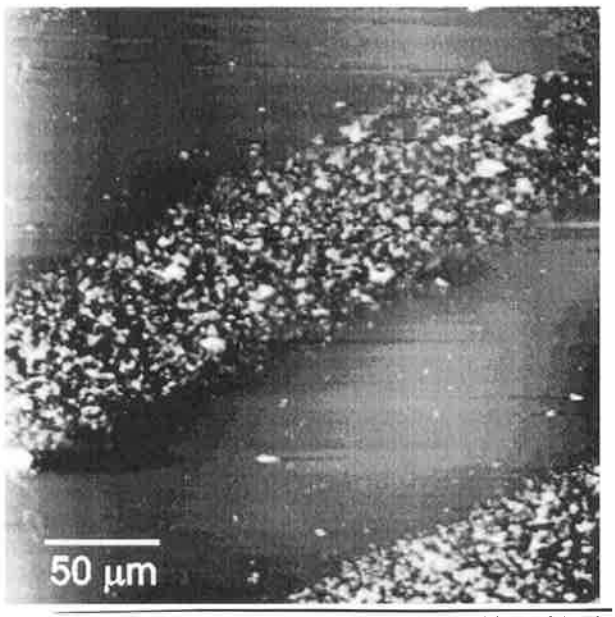

Fig. 4. Mask-assisted photopatterning on silicon nitride with T-BSA. Phc tolabel-derivatized bovine serum albumin (T-BSA) was spotted on silicon nitride and dried, covered with a photomask (slit size 20 μm) and irradiated for 20 min (0.7 mW cm^{-1}) at 350 nm. After removal of excess T-BSA, the surface was imaged by scanning force microscopy in sodium phosphate buffer.

investigations indicate that silicon nitride in aqueous media is highly oxidized (silanol) and contains less than 1% amine sites [9]. The availability of surface hydroxyl functions is essential for thermochemical functionalization (CNBr, silylation) of silicon nitride surfaces, and subsequent covalent immobilization of proteins [10]. Complementary to such thermochemical surface modifications, photochemistry enabled facile functionalization of silicon nitride with biomolecules. Aryldiazirin-based immobilization procedures were formerly applied for bioengineering of numerous materials differing in chemical composition and surface reactivity [11]. As shown in this study, direct and indirect carbene-based covalent functionalization of silicon nitride can be achieved with a variety of biomolecules. Biological activities were recovered on bioengineered silicon nitride, including antigen binding to oriented F(ab') fragments.

4. Conclusions

The study identifies silicon nitride as an attractive material for spatially selective bioengineering. Both single-step and two-step immobilization procedures are applicable, enabling locally selective and oriented biomolecule binding. Such procedures are essential for the distinct immobilization of biomolecules on sensor surfaces. The process of photoimmobilization is experimentally facile and confers with photolithographic procedures applied in microstructuring technologies. To date, silicon nitride has been advantageously used in microelectronics, optics, scanning probe technology and medicine (implantation surgery). In conjunction with light-dependent surface functionalization, silicon nitride offers a plethora of new applications, including the construction of biosensors and bioelectronic devices.

Acknowledgements

The competent assistance of Mrs O. Bucher is gratefully acknowledged. The work was financially supported by the Swiss Priority Program on Biotechnology and the Kommission für Technologie und Innovation, Bern.

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